In certain cartilaginous fish there is a combination of both of these arrangements of tubes, from the dentine and from the surface, and sundry other apparently anomalous conditions are met with.

But if the views advocated in this paper be accepted, all difficulty in accounting for these arrangements, very difficult to explain from any teleological standpoint, disappear, for they become merely slight variations or arrests at different stages of a process common to all enamels during their formation.

"On a Green Leucocytosis in Oysters associated with the presence of Copper in the Leucocytes." By Rubert Boyce, M.B., Professor of Pathology in University College, Liverpool, and W. A. Herdman, D.Sc., F.R.S., Professor of Zoology in University College, Liverpool. Received July 9, 1897.

In the course of an investigation upon oysters under normal and abnormal conditions, upon which we have been engaged for the last two years, and upon which we propose to submit to the Society a detailed memoir during next session, we have come upon a phenomenon which we regard of such considerable importance that we desire to publish a brief record of our observations and experiments, as we believe they may prove of interest to other biologists who are engaged in work on the micro-chemistry of the cell. The phenomenon we have now to describe is the presence of large quantities of copper in certain green leucocytes found in a diseased condition of the American oyster. The oysters suffering from this leucocytosis are always more or less green, but must not be confounded with ordinary green gilled oysters, where the colour is due to a totally distinct cause.

History.

Green oysters have been known from an early period, and there are various historic cases on record* of people having been poisoned by eating green oysters, and of the oyster merchants being put upon trial because of the deleterious nature of their goods. Periodically green oysters have been suspected or convicted of being coloured with copper, and just as often it has been proved by competent authorities that copper has nothing whatever to do with the green colour. This difference of opinion in the past has undoubtedly been

* An interesting historical survey of the subject up to 1866, was given by the late Mr. Arthur O'Shaughnessy, in the 'Annals and Mag. Nat. Hist.,' ser. 3, vol. 18.

largely due to the fact that the observers worked with different kinds of oysters. Some have investigated the celebrated "Huîtres de Marennes" (a form of *Ostrea edulis*), and have found that while having dark blue-green gills, they were still in a perfectly healthy state, that they contained very little copper, and that some iron was present in the pigment. All that is perfectly correct, but it does not enable us to draw any conclusions in regard to other green oysters. There are evidently several kinds of greenness in oysters, and whereas some may be due to normal and healthy processes, others must be regarded as abnormal or diseased conditions. It is the latter, in our experience, that contain the copper.

As early as 1835, Bizio showed that certain oysters he obtained at Venice contained copper, and he attributed (1845) their bluish-green colour, and that of the Marennes oyster, to the presence of that metal. Two subsequent discoveries have thrown a certain amount of probably undeserved discredit upon Bizio's work. These are (1) the determination by Fredericq and others that a certain small amount of copper is present normally in the hæmocyanin of the blood of crustaceans and molluscs; and (2) the excellent work of Lankester* and others on the Marennes oysters which established the normal, healthy condition of the greenness, and the absence in that form of any copper beyond the trace due to hæmocyanin. We now think it very probable, in the light of our recent experience, that Bizio was dealing, in the case of his Venetian oysters, with the same copper-bearing green pigment that we have met with.

About 1880 Ryder investigated some green oysters in America, and from his description of what he found we cannot doubt that he had before him the same kind of green American oyster (*Ostrea virginica*) that we have been examining. He showed that the green colouring matter was taken up by the amœboid blood cells, and that these wandering cells containing the pigment were to be found in the heart, in some of the blood vessels, and in aggregations in "cysts" under the surface epithelium of the body. He describes the colour (in the ventricle) as a "delicate pea-green," and states that it is not chlorophyll nor diatomine: he suggests that it may be phycocyanin or some allied substance.†

So far as we are aware there has been no work‡ since Ryder's,

* See Professor Lankester's memoir on "Green Oysters," in the 'Quart. Jour. Micro. Sci.' for 1886, which gives an excellent discussion of the subject so far as the Marennes oyster is concerned.

† Ryder's papers are in the 'U.S. Fish. Commission Reports and Bulletins' rom 1882 to 1885.

‡ Except Carazzi's passing allusions to our work in 'Mitth. Zool. Stat. Neapel' for 1896. His own investigations were made upon other kinds of oysters.

dealing with what we described a couple of years ago as the green leucocytosis in the American oyster. Many papers, to which we do not refer, have appeared dealing with other kinds of green oysters, but they do not affect our present subject.

In January, 1896,* we referred briefly to what appeared to be an inflammatory condition, accompanied by a pale chalky-green colour, which we found in some American oysters relaid at Fleetwood, on the Lancashire coast; and at the Liverpool meeting of the British Association, last September, in discussing various kinds of greenness in oysters, we referred to this diseased condition, in the following terms:—

"There is, however, a pale greenness (quite different in appearance from the blue-green of the "Huîtres de Marennes") which we have met with in some American oysters laid down in this country, and which we regard as a disease. It is characterised by a leucocytosis, in which enormous numbers of leucocytes come out on the surface of the body, and especially on the mantle. The green patches visible to the eye correspond to accumulations of the leucocytes, which in mass have a green tint. These cells are granular and amœboid. The granules do not give any definite reaction with the aniline stains, and, so far, we have not made out their precise nature."

Finally, towards the end of last year, in the 'Supplement to the Twenty-fourth Annual Report of the Local Government Board,' Dr. Bulstrode corroborated our statement as to the presence of the pale green disease from the examination of specimens from Truro and Falmouth. Dr. Thorpe stated in the same Report that he had found that some green oysters from Falmouth,† sent to him for examination by Dr. Bulstrode and Mr. R. Vallentin, contained notable amounts of copper, in some cases as much as 0·02 grain per oyster, while the amount normally present is only 0·006 grain.

Dr. Charles Kohn has kindly, during the last year or so, made a number of analyses for us of different kinds of oysters—"natives," "Marennes," Dutch, and American—and whereas in most of these he has found the copper to be present only in small quantities, on the average agreeing well with the amount (0·006 grain) usually stated as present in the tissues of the normal healthy oyster, in some green Americans which we gave him recently for the purpose he has found a very much larger amount of copper. These circumstances induced us to reopen, in our investigations, the question of copper in certain green oysters, with the results that are detailed below.

* Report for 1895 on the Lancashire Sea-Fisheries Laboratory ('Trans. Biol. Soc.,' Liverpool, vol. 10, p. 158).

† Obtained from a creek, which is locally supposed to bring down copper, and the water of which was found on analysis to contain some copper.

The Green Leucocytosis.

We first noticed this diseased condition in the autumn of 1895, in some ordinary American oysters ("blue points"), belonging to the species *Ostrea virginica*, which had been imported into Liverpool and relaid near Fleetwood, in the estuary of the Wyre. Since then many hundreds (probably several thousands) of American oysters have been examined by us, and we have seen all degrees of the leucocytosis. It manifests itself in patches and streaks of green on the mantle and other parts of the integument, in engorgements of the blood vessels, especially of those that ramify over the surface of the viscera, and in masses of green-coloured leucocytes in the heart. This green condition, although much less frequently seen in "natives" (*O. edulis*), is occasionally met with there also, and we have recently had some specimens from Falmouth with very well-marked green hearts, due to an accumulation of leucocytes laden with green granules in the ventricle. Such hearts are of frequent occurrence in the diseased American oysters; after death the mass of leucocytes subsides to the bottom of the cavity, leaving the clear plasma above. It is thus easy to demonstrate that the colour is due to the leucocytes, and to the leucocytes alone.

The blood of these oysters contains a great variety of more or less colourless and more or less green and granular corpuscles, all of which may be termed leucocytes. They are apparently all amœboid wandering cells, comparable to the colourless corpuscles of the blood of higher animals. The larger and (probably) older of the leucocytes are very coarsely granular and very opaquely green. It is these that give the colour in bulk. We find them in masses in the heart, in both auricle and ventricle, in the vessels, where they are sometimes so abundant as to engorge or inject certain parts of the system, in the lacunar spaces of the connective tissue of the mantle and other organs, and also in the more solid parts of the tissues wandering amongst the other cells, wedged into the epithelium and coming out in great numbers on the surface of the body. Some of these latter, when found in the ectoderm and on the surface, are very markedly eosinophilous; those in the vessels are not so markedly so. When stained with osmic acid the granules of the leucocytes become black. After treatment with fat solvents, however, some of the leucocytes are still very granular.

In sections which have not been stained, the granules of the leucocytes have a distinctly brown colour, recalling the appearance of the granules in the liver cells in unstained sections in cases of pernicious anæmia.

We opened many batches of American oysters, 100 at a time, and in all cases where the green tint was present in the mantle, heart, or

vessels we found the accumulations of leucocytes. From 120 oysters we chose the six greenest and the six whitest. Dr. Kohn analysed these two sets of six for us, and found that the green contained between three and four (3·7) times as much copper as the white. This shows that it is not merely a redisposition in the body of the copper, due possibly to the hæmocyanin, but that there is an absolute increase in the amount present in the body.

We also found that the greenest parts of the body contained far more copper than corresponding tissues which had no green deposit in them. Not only then do these green oysters contain a largely increased amount of copper, but we have also shown that the copper coincides in its distribution with the green leucocytes, and, consequently, we regard the copper as the cause of the green colour. We then passed on to a more minute examination of the pigment and to histo-chemical reactions.

Chemical Reactions.

The Green Colouring Matter.—The greenest portions of the green oysters were snipped out and dried on the water bath. The dried powdered residue was treated with alcohol, ether, chloroform, benzene, turpentine, xylol, but these reagents failed to extract the colouring matter; we concluded, therefore, that the pigment was not of the nature of a lipochrome. On the other hand, the pigment was readily soluble in dilute acids and in alkalis; the addition of ammonia gave rise to a distinct bluish tint, and fresh pieces of the green oysters reacted instantly with ammonia, with the formation of a beautiful blue.

We next determined whether the pigment was due to iron or copper. The dried residue treated with dilute hydrochloric acid and potassic ferrocyanide gave a marked red reaction, thus indicating the presence of copper, and it was then found that very small quantities of the green colouring matter treated with dilute hydrochloric acid were sufficient to produce a well-marked deposit of metallic copper upon polished iron. In several instances a deposition of copper occurred when a piece of polished iron was laid upon a green patch on the surface of the mantle of a fresh oyster, dilute hydrochloric acid having been previously used to moisten the mantle. Control experiments were made with the whitest portions of the American oysters and with natives, and traces only of copper were found. These results have been also quantitatively controlled by Dr. Kohn.

A series of histo-chemical reactions were then carried out. For the purpose the oysters were hardened in absolute alcohol, and pieces were then imbedded in paraffin, great care being taken that

every reagent was perfectly pure, firstly, with regard to the absence of copper or iron, and, secondly, that no acid was present; thus, for example, commercial turpentine may give a distinctly acid reaction, and this would be sufficient to remove the copper. If sections were imbedded in gum—and often the best results were obtained by this method—the tissues were allowed to remain for as short a time as possible in distilled water and then transferred to perfectly fresh neutralised solution of gum-arabic, and allowed to remain in it for only a short period. The pigments appeared partially soluble in water.

Comparatively thick sections were cut, in which the distribution of the green colour could be seen with the naked eye. These were placed in absolute alcohol in every case before proceeding to test. The reagents which we employed were potassic ferrocyanide, 1·5 per cent. solution,* freshly prepared ammonium-hydrogen sulphide, and pure hæmatoxylin.

Potassic Ferrocyanide.—Sections were taken from absolute alcohol and passed into distilled water for a moment in order to remove the alcohol. They were then placed in the potassic ferrocyanide solution, when the portions previously green assumed a red colour; this reaction set in immediately. The presence of a 0·5 per cent. solution of hydrochloric acid added in equal quantity to the ferrocyanide solution previous to use (as recommended by Macallum for iron) tended to hasten the reaction, and in some cases was necessary in order to obtain it.

The sections were then washed in distilled water, dehydrated in absolute alcohol, cleared in cedar oil, and mounted in Canada balsam. The red coloration was found located to the masses of leucocytes, and the individual leucocytes themselves were of a faint yellowish-red colour. In the cases of the very granular pigmented leucocytes the granules assumed a distinct red-brown colour. In this way the distribution of the leucocytes and of the vessels which contained them was mapped out. Very beautiful preparations of the engorged green vessels were obtained by partially dissecting the mantle in the fresh oyster so as to expose the ramifying vessels, then hardening in alcohol, and subsequently treating with ferrocyanide solution, when the vessels assumed a well-marked red colour; beautiful results were also obtained by ammonia. Fresh blood obtained from the heart in which vast numbers of the green leucocytes were present also gave a red reaction with acidulated ferrocyanide solution. Control bloods from white oysters gave an exceedingly faint or no reaction.

* For the sake of uniformity we finally adopted the strength of solutions given by Macallum in his paper on the "Distribution of Assimilated Iron Compounds," 'Quarterly Journal of Microscopical Science,' 1896.

Ammonium-hydrogen Sulphide.—Sections taken out of the alcohol and placed in this solution instantly gave a marked dark yellow-brown reaction wherever there were green patches. This reagent is more striking in its results than the potassic ferrocyanide, and very good cover-slip preparations of the blood can be obtained, the corpuscles staining dark yellow-brown.

Hæmatoxylin.—We were led to use this reagent from knowledge of its reaction in the case of Weigert's nerve-staining method. The results are most striking. Sections placed in a watch-glass of distilled water, to which a few crystals of pure hæmatoxylin are then added, begin at once to assume a distinct blue colour in the place of the previous green; this occurs whilst the solution itself remains free from colour, and therefore whilst the quantity of hæmatoxylin dissolved must be very minute. Microscopic examination shows the corpuscles dark blue, and the vascular network beautifully differentiated. The connective tissue and gland cells and nuclei remain unstained, or occasionally show a very faint blue reaction, most marked immediately around the vessels. This reaction appears to us to be as specific for copper as Macallum showed it to be in the case of inorganic iron. Just as in the test-tube, so in the cell, a blue-black reaction is obtained not only with iron (as in the state seen in the liver cells in pernicious anæmia) but also with copper. It therefore follows that hæmatoxylin is a most sensitive test for either metal, and that consequently in the outset it is necessary to determine whether copper or iron is present exclusively in the cells, and to which of these elements the reaction is due.

Iron is found in the ash of the oyster, and the green coloration of the Marennes oysters has been attributed to it by Carazzi and others. In the case of the green oysters which we have examined, Dr. Kohn found, in addition to the copper, traces of iron—the iron was, however, far below the copper in quantity. In the detailed and valuable paper of Macallum, previously referred to, a series of histo-chemical reactions are described in order to demonstrate the presence of iron in cells, and he with others distinguishes two forms, organic and inorganic. The latter, like, we presume, the iron in the liver cells in pernicious anæmia,* gives an immediate reaction with potassic ferrocyanide and dilute hydrochloric acid, and as Macallum has shown,† a dark blue with pure hæmatoxylin. But the organic iron behaves differently, giving, according to Macallum, a yellow colour with hæmatoxylin, and requiring previous treatment with dilute nitric, sulphuric, or hydrochloric acids in alcohol before a Prussian blue reaction is obtained with acidulated potassic ferrocyanide, or prolonged

* We have obtained an immediate blue reaction with hæmatoxylin in the liver in five cases of pernicious anæmia.

† 'Report British Association,' Liverpool, 1896, p. 973.

treatment with ammonium sulphide before any dark coloration is obtained with that reagent. Now it will be observed that all our reactions were *immediate*, taking place directly on the addition of ammonium sulphide, or potassic ferrocyanide alone, or aided by a trace of acid. The copper was therefore present in a condition analogous to the inorganic iron, or at least so loosely combined with the cell protoplasm as to be readily discharged, but in none of these cases did we get any indication of inorganic iron, except in the case of the *contents* of the alimentary tract of the oyster. When the sections were treated with 3 per cent. nitric acid in alcohol for half an hour the green colour disappeared, and then neither the copper reaction nor the striking reactions with ammonium sulphide and hæmatoxylin took place. Subsequent treatment of these sections with acidulated potassic ferrocyanide, and again washing in dilute nitric or hydrochloric acid, yielded a general and very faint Prussian blue reaction, in which the nuclei of the gland cells were more markedly blue than the leucocytes. If the method is reliable it shows that traces of iron are present in the cells in addition to the copper, but it is the organic iron. Some oysters gave this Prussian blue reaction more markedly than others; this was the case with some Falmouth "natives."

Hæmocyanin containing copper has been shown to be an important constituent of the blood in many of the invertebrata, taking the place of hæmoglobin. We have examined the blood of very many oysters, and only in two instances, and these in green oysters, have we thought that the plasma became very faintly blue on exposure to oxygen, whilst, as previously indicated, qualitative tests either failed to give any indication of copper or, at most, only a very faint reaction, and even in these cases the reaction appeared confined to the leucocytes which were present in the plasma. The ash, however, of the white oyster yields about 0·006 grain of copper, and it is probable that minute traces are present in the plasma as hæmocyanin. The cause, then, of the presence of the copper in such abundance in the green leucocytes is very obscure. The quantity of copper in the green leucocytes themselves varies, as our histochemical reactions demonstrated; some corpuscles could be found which were conspicuous by their red reactions on the addition of potassic ferrocyanide, whilst others in the same preparations only gave very faint indications, and occasionally a cell could be seen which gave a marked Prussian blue reaction instead of the red.

Conclusions.

Our results demonstrated the presence of copper in comparatively large quantity in the green leucocytes, chiefly in the American oyster, but also in the "natives" from Falmouth and other localities.

We have shown that the colour was in proportion to the amount of copper present, and that the colourless leucocytes contained only traces of that metal. The deposition of the copper in this large quantity appears to us to represent a degenerative reaction; it was accompanied by a most striking increase of leucocytes, which tended to distend the vessels and to collect in clumps, phenomena which are abnormal in our experience in the oyster. The presence of the copper in the leucocytes in these cases might be compared to that of the iron which is met with in some of the leucocytes in cases of old hæmorrhages, pernicious anæmia, or in other cases where iron is set free. We are not prepared to state whether copper in the food can bring about the condition, but certainly we have abundant evidence to show that it can occur where no copper mines or other evident sources of copper are present.

We are inclined to suggest that the increase of copper may be due to a disturbed metabolism, whereby the normal copper of the hæmocyanin, which is probably passing through the body in minute amounts, ceases to be removed, and so becomes stored up in certain cells.

Our results also show that hæmatoxylin is a most valuable reagent, not only as Macallum has shown in the case of iron, but also in that of copper, and that care must be taken to distinguish between the two reactions; and this must be especially the case in those invertebrata where copper plays an important *rôle* in the physiology of the blood.

"Stress and other Effects produced in Resin and in a Viscid Compound of Resin and Oil by Electrification." By J. W. Swan, F.R.S. Received May 17,—Read June 17, 1897.

(Plates 1—4.)

While making an experiment with the object of finding the degree of resistance to puncture offered by paper coated with a soft compound of resin and oil, when placed between the secondary terminals of an induction coil, the tension being regulated by a spark-gap in a parallel branch of the circuit, observed that on the passage of a spark at the spark-gap, while no spark passed between the paper-separated terminals, a sudden roughening or puckering of the previously smooth surface of the coating took place.

A number of experiments were made with the object of ascertaining the nature of the action which produced this effect, and these led to further experiments and to results which, though closely related to well-known phenomena, possess features of novelty and interest.